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(71) Applicant (for all designated States except US): WISCONSIN ALUMNI RESEARCH FOUNDATION [US/US]; 614 Walnut Street, Madison, WI 53705 (US).

(72) Inventors; and
(75) Inventors/Applicants (for US only): HULLETT, Debra, A. [US/US]; 546 Orchard Drive, Madison, WI 53711 (US). ALAM, Tausif [US/US]; 310 University Houses, Madison, WI 53705 (US). SOLLINGER, Hans, W. [DE/US]; 1202 Sherman Avenue, Madison, WI 53703 (US). THERON, Amy, L. [US/US]; 614 Walnut Street, Madison, WI 53705 (US).

(74) Agent: BARTA, Kent, S.; Michael Best & Friedrich LLP, 100 East Wisconsin Avenue, Milwaukee, WI 53202–4108 (US).

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(54) Title: TREATMENT OF DIABETES WITH SYNTHETIC BETA CELLS

Insulin Expression Clones in Adenovirus

GIRE* Rat Albumin Promoter   Modified Human Insulin cDNA

NotI   EcoRI   KpnI   SalI

C (80 bp)   A (195 bp)   M1/M2 (430 bp)
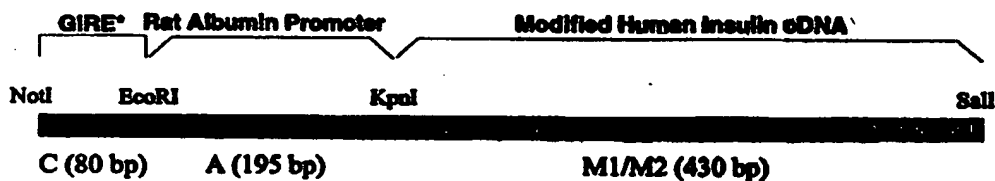
(57) Abstract

Hepatocytes transfected with a replication deficient adenovirus vector containing a gene cassette expressing the proinsulin gene in response to physiological levels of glucose provide novel beta islet replacement cells. The cassette comprises the structural gene for human proinsulin genetically altered to make it cleavable to active insulin, a promoter operably linked to the proinsulin gene, and a glucose regulatory response module located 5' to the promoter. Synthesis of proinsulin mRNA is ablated at less than 5mM glucose, and peaks at about 15 mM.

TREATMENT OF DIABETES WITH SYNTHETIC BETA CELLS

CROSS REFERENCE TO RELATED APPLICATIONS

This application is a continuation-in-part of co-pending application Serial Number 08/786,625 filed January 21, 1997.

FIELD OF THE INVENTION

This invention relates to the field of gene therapy and to a method of utilizing normal non-islet cells transfected with a proinsulin gene inducibly expressed in such cells in the presence of glucose. The proinsulin synthesized in the cells is further processed into mature insulin.

BACKGROUND OF THE INVENTION

Insulin-dependent diabetes mellitus (IDDM or Type I diabetes) occurs when an autoimmune response destroys the beta cells of the islets of Langerhans, resulting in cessation of insulin production. For many years, and indeed for many patients even at present, the only recourse for treating this fatal condition is the periodic administration of injectable insulin of animal, or more recently, of recombinant human origin. While the administration of exogenous insulin is life-saving over the long term, severe side effects, such as circulatory disturbances resulting in blindness, gangrene, and heart attack are common. The doses of insulin injected into the diabetic patient are only approximate, even when careful dietary controls are implemented. These continual imbalances in blood glucose resulting from deviations from optimal levels of insulin are thought to cause, or contribute to, the observed side effects.

There have been many attempts to minimize the side effects of insulin therapy. Home blood and urine glucose test kits help the diabetic monitor blood sugar

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levels. Some researchers have proposed pump devices
which meter insulin continuously and in more precise
doses. One interesting such device is disclosed in
U.S. Patent No. 5,364,838 which delivers insulin in
5   aerosol form. The hormone is absorbed through the
lung, thereby avoiding the more invasive injection
route of administration.

In the field of diabetes there is a general
recognition that replacement of beta cellular function
10   would be a superior therapy to insulin administration,
because the natural cell would secrete insulin in
response to glucose levels in the microenvironment.
This fine-tuned control would thereby eliminate the
deleterious side effects of insulin administration.
15   Indeed, it has been shown in the relatively small group
of patients who have successfully received pancreatic
transplants that remission of side effects or cessation
of progressive tissue damage results. Unfortunately,
pancreatic transplant is available only to a few
20   diabetics, compared to the numbers of afflicted
persons. In addition, transplantation is associated
with significant toxicity due to immunosuppressive
therapy.

There have been many alternatives proposed for
25   providing the benefits of beta cell replacement without
involving organ transplantation. These alternatives
involve replacement of beta cell function with actual
beta cells or other insulin secreting pancreas-derived
cell lines, as discussed in Lacy, et al., Ann. Rev.
30   Med., 37:33 (1986). Since introduction of exogenous
cells into the body is perceived by the immune system
as any other allograft, it is necessary to isolate the
cells from contact with immunoactive cells and
substances. In particular, donor islet cells must be
35   protected from T-cells and macrophages mediating
cytolytic processes. One approach is physical
immunoisolation, either by microencapsulation or by a
microporous chamber. The challenge in these

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technologies is to overcome the natural process of foreign body rejection resulting in walling off the implant by a dense layer of fibroblasts. The key to a successful implant is to provide both a biocompatible surface and sufficient porosity to permit some degree of microvascularization. For a general review of these approaches, see Brauker, et al., J. Biomed. Mat. Res., 29: No. 12, 1995. To date, immunoisolation still poses significant problems including loss of viability and function of captive cell populations, foreign body rejection, fissure and inflammation, and immune reactions.

Another approach is to engineer beta cell function into cells derived from autologous tissue or artificially constructed cell lines. U.S. Patent No. 5,427,940 discloses an artificial beta cell produced by engineering endocrine cells of the At-T-20 ACTH secreting cells. A stably transfected cell At-T-20 ins is obtained by introducing cDNA encoding human insulin and the glucose transporter gene GLUT-2 driven by the constitutive CMV promoter. The cell line already expresses the correct isoform of glucokinase required for glucose responsive expression of the insulin gene. This cell line is responsive to glucose, but is regulated at a level of secretagogue below physiological range. Hence, while the system is of interest, it is not of clinical significance because an animal into which these cells are introduced would be chronically hypoglycemic. Another disadvantage is that the cells, being derived from a heterologous source, bear their distinctive foreign antigens, and must be used in immunoisolation. A further disadvantage is that At-T-20 is a transformed cell line with potential for unlimited growth.

U.S. Patent No. 5,534,404 discloses another approach to obtaining a correctly secretagogue regulated cell line. Starting with beta-TC-6 cells, subpopulations of cells are selected in an initial

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stage by a cell sorter capable of recognizing cells
having an increased internal concentration of calcium
ion, associated with insulin expression (Ca++ activated
fluorescence). After successive passages, cell

5   populations are further selected which respond to
glucose in the physiological 4 to 10mM range in a
typical sigmoidal curve. For therapeutic use, the
cells were encapsulated in alginate bounded by a
PAN/PVC permselective hollow fiber membrane according

10   to the method of Dionne (U.S. Patent application No.
PCT/US92/03327).

        Valera et al., FASEB Journal, 8: 440 (1994)
describes transgenic mouse hepatocytes expressing
insulin under control of the PEPCK promoter driven by

15   P-enolpyruvate. The PEPCK promoter is sensitive to the
glucagon/insulin ratio and is activated in elevated
glucose states. The PECK/insulin chimeric gene was
introduced into fertilized mouse eggs. Under
conditions of severe islet suppression by

20   streptozotocin, the production and secretion of intact
insulin by the liver compensated for loss of islet
function.

        The strategy of gene therapy for treatment of
diabetes is complicated by the complexity of insulin

25   regulation and the structure of the protein itself.
The glucose responsive release of insulin from the beta
islet cells is a complex event involving migration of
preprocessed protein from cytoplasm to the Golgi
apparatus where secretory granules bud off and travel

30   to and fuse with the plasma membrane prior to release.
The initial protein product is preproinsulin having an
N-terminal signal sequence, which is cleaved during
transport to the rough endoplasmic reticulum.
Thereafter the resulting proinsulin is further

35   processed to insulin by removal of the C-peptide
joining the two polypeptides of the mature molecule,
the A and B chains. In engineering the production of
insulin in a host cell, it is impossible to obtain a

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functional insulin by merely providing the A and B
polypeptides. Synthesis of an intact molecule is
necessary for proper folding, and only after the
correct conformation is obtained, can the C-peptide be
5   snipped out. Thus, any engineered cell expressing
mature functional insulin must have the Kex2 enzyme
machinery, including the PC1/PC3 and PC2
endopeptidases, or a functional substitute thereof, as
suggested by Newgard, Biotechnology, 10: 1112 (1992).
10   The control of insulin production and release is
further complicated by the regulation of glycolytic
flux. It is believed that two proteins are used by the
beta cell to sense changes in glucose levels: Glut-2 a
specific facilitated diffusion type glucose
15   transporter, and a particular glucose phosphorylating
enzyme, glucokinase IV. Both enzymes have a higher Km
and Vmax than the other enzymes in their related
families. Both also have high affinities for glucose
that result in large shifts in activity over the
20   physiological range of glucose concentration. While
reduction in GLUT-2 results in depression in insulin
production, loss of glucokinase abruptly halts insulin
production, and identifies glucose phosphylation as the
true rate limiting step. Transformation of cells with
25   expressible genes for these enzymes appears to restore
glucose responsive regulatory characteristics to
insulin production, but not infrequently outside the
physiological range of control. The experiences many
researchers have had underscores the problems inherent
30   in the complexity of the control of insulin production
through manipulation of the metabolic utilization of
glucose. There is thus a need in the field of diabetes
for a new model of insulin regulation and beta cell
replacement.
35

SUMMARY OF THE INVENTION

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Control of insulin production in synthetic beta cells may be accomplished by alternative regulatory pathways than through attempted restoration of natural control over a transformed beta cell expression system. While the actual release of insulin in normal beta cells is modulated through metabolic intermediates, as yet poorly understood, an alternative control is at the level of transcription of the mRNA encoding the proinsulin precursor. It is thus an object of the present invention to provide a control system for expression of the proinsulin gene in a suitable host cell, which is independent of the metabolic effectors and intermediates involved in normal regulation.

It is a further object to provide a replacement beta cell autologous with the patient's own cells to avoid a requirement for immunoisolation of insulin-producing cells. Ideally, a cell population is to be selected which can be engineered to synthesize insulin dependent on regulated gene transcription, without excision and extracorporeal manipulation outside the body. It is a further object to provide a gene therapy utilizing a cell population having intact and normally functioning glucose transporter and phosphorylating system, so that control of insulin production is a function only of transcriptional control. Consistent with this object is provision of an enzyme system capable of generating active insulin or insulin-like analog from proinsulin not subject to feedback intervention of the glycolytic pathway.

In accordance with the present invention, a gene cassette for expression of proinsulin in autologous host cells comprises a nucleotide sequence coding substantially full length proinsulin DNA, most preferably insulin cDNA, operably linked to a promoter recognized by an RNA polymerase contained in the host cells, together with a glucose responsive regulatory module having at least two glucose inducible regulatory elements located upstream at the 5' end of the

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promoter. The cassette is integrated into a vector
comprising a replication defective viral genome
capable, when infecting a suitable target cell in
vitro, of packaging the vector in a viral particle
5   infective for the autologous host cells. The preferred
target host cell is the hepatocyte because liver cells
already express GLUT-2 and glucokinase IV sufficiently
to generate the appropriate intermediates for glucose
regulated transcriptional control of the proinsulin
10   gene in the physiological range.

        Hepatocytes also express the endopeptidase furin.
A mutation can be introduced into the reading frame of
the proinsulin gene that permits furin cleaving at the
appropriate site to obtain substantially complete
15   excision of the C-peptide with appearance of
essentially native insulin activity. It is therefore
an aspect of the present invention that in the
transfection method, a vector is provided in which
transcriptionally controlled production of proinsulin
20   is substantially completely converted to the active
hormone, which is constitutively secreted into the
liver parenchyma in response to elevation in glucose
concentration.

25   BRIEF DESCRIPTION OF THE DRAWINGS

        Figure 1 is a schematic depicting the nucleotide
sequence of the glucose regulatory modules C and F
respectively.

30

        Figure 2 is a genetic map of the pACCMV.plpA 8.8
kb plasmid containing the cloning sites for the
expression cassette for proinsulin, and also several
adenoviral genes.

35

        Figure 3a is a genetic map showing the insertion
diagram of the expression cassette in relation to

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various markers on the pACCMV.p.A plasmid. Figure 3b
shows the order of genetic elements 5' to 3'.

5   Figure 4 is a genetic map of the large 40.3 kb
pJM17 plasmid used to create the final recombinant
vector for transfection.

Figure 5 is a genetic map showing the
recombination of vectors pACCMV.plpA and pJM17 to yield
10   the AdC/FAM construct.

Figure 6 is a gel reproduction of a Northern blot
of RNA isolated from hepatocytes transfected with the
recombinant plasmid vector containing the expression
15   cassette, and cultured in the presence of various
levels of glucose.

Figure 7 is a gel reproduction of a Northern blot
identical to figure 6, only showing the result of a
20   longer exposure of the CMV control.

Figure 8 is a duplicate experiment of that
depicted in Figure 6 only showing the migration
position of a control band of mRNA under control of the
25   constitutive CMV promoter.

Figure 9 is a gel reproduction comparing rRNA
bonding with mRNA from hepatocytes in the presence of
various levels of glucose.
30
Figure 10 is a schematic diagram of the steps used
to clone M2A and M2B.

Figure 11 is a graph comparing the amount of
35   insulin released from Cos7 cells transfected with
recombinant plasmids.

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Figure 12 is a graph comparing the secretion and intracellular retention of insulin in Cos7 cells transfected with recombinant plasmids.

5   Figure 13 is a autoradiograph demonstrating the effect of time on glucose inducible upregulation of hIns mRNA in transfected hepatocytes. (Lanes 1,2, and 3- hIns mRNA expression at 27.5 mM glucose for .5h, 1h, and 16h, respectively. Lanes 4, 5, and 6-hIns mRNA

10   expression at 5.6 mM glucose for .5h, 1h and 16h, respectively.)

Figure 14 is an autoradiograph demonstrating that glucose responsive human insulin production is observed

15   in cell lysates from transduced rat hepatocytes. (Cells were transfected with the glucose inducible construct (Lanes 1 and 2) and cultured in 3.3 mM glucose (Lane 1) or 27.5 mM glucose (Lane 2). Lane 3 is a control lane of cells transfected with proinsulin

20   under the control of a CMV promoter. Lane 4 represents the same material as in lane 3, except that an excess of unlabelled insulin was added prior to the immunoprecipitation step with insulin specific antibody.)

25

Figure 15 is a bar graph comparing insulin produced by hepatocytes transfected with a construct containing 1 GIRE at different glucose concentrations.

30   Figure 16 is a bar graph comparing insulin produced by hepatocytes transfected with a construct containing 2 GIREs at different glucose concentrations.

Figure 17 is a bar graph comparing insulin

35   produced by hepatocytes transfected with a construct containing 3 GIREs at different glucose concentrations.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

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In the present invention, replacement beta cells for treatment of diabetes Type I are constructed by transfection of autologous cells, preferably hepatocytes, with a vector expressing proinsulin
5   genetically modified to be cleavable to insulin by an enzyme or enzymes endogenous to the transfected cells. Initially, a gene cassette is constructed containing the proinsulin gene and control elements suitable for its expression regulated by a secretagogue, preferably
10   glucose.

In the isolation of native proinsulin cDNA, total RNA from normal human islet cells was extracted, and the mRNA fraction was isolated and used as a template in an oligo $(dT)_{15}$ primed reverse transcription
15   reaction. Insulin cDNA (-28bp-443bp) was amplified using sense and antisense oligonucleotides which included restriction sites for KpnI and SalI, respectively. The sequences are shown in Table 1 designated TA423 and TA413, and are listed herein as
20   SEQ ID NOS: 1 and 2. Alternatively, cDNA can be isolated according to the methods described in Bell, et al., Nature, 282: 525 (1979) using the primers disclosed therein, but incorporating restriction sites compatible with the selected cloning vehicle. In
25   general, it is desirable to include in the amplified region a portion of the intron flanking the open reading frame of the proinsulin gene. The amplified DNA fragment (-28-443bp) containing the entire coding sequence of human insulin and a portion of the
30   untranslated region from the 5' and 3' ends was subcloned into pBlueScript SK+. pBlueScript SK+ is a 2.96 kb colony-producing phagemid derived by replacing pUC19 polylinker of pBS(+/-) with a synthetic polylinker. This cloning vehicle is described in Shon,
35   et al., Nuc. Acids Res., 16: 7583 (1988).

In a preferred embodiment of the invention, hepatocytes are transfected with a vector having a glucose regulated proinsulin gene. Hepatocytes express

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an endogenous endopeptidase furin. Although furin is
known to cleave proinsulin at its B-C junction, it is
very inefficient at cleaving the C-A junction.
Cleavage at both sites is required for excision of the
5   C-peptide required for conversion of proinsulin to
active insulin. A single point mutation (T^{267} to G
converts the amino acid sequence KQKR to RQKR producing
a modified C-A junction compatible with the specificity
of furin. Thus, the proinsulin protein can be
10   processed to insulin utilizing a single endogenous
enzyme.

The mutation creating the new C-A junction may be
effected by standard methods known in the art. For
example, conversion of Lys to Arg can be made in two
15   steps. The sense oligonucleotide (TA403 designates SEQ
ID NO: 3) including a point mutation corresponding to
the desired change in the target region was used with
the original insulin antisense oligonucleotide (TA413)
to amplify one segment of insulin. Similarly an
20   antisense oligonucleotide (TA404) containing the Lys to
Arg mutation was used with the original insulin sense
oligonucleotide (TA423) to amplify the second fragment
of modified insulin (M1). The two fragments, thus
produced, can be purified, and a mixture of them used
25   as template DNA in amplification of C-A modified
insulin M1 with oligonucleotide TA423 and TA413. The
C-A modified insulin M1 may be subcloned in pBlueScript
SK+ at the restriction sites Kpn I and Sal I. In
practice, the InsM1 DNA was sequenced and found to be
30   error free. TA413, TA404, and TA423 are shown in Table
1, and are listed herein as SEQ ID NOS: 2, 4 and 1.

The key aspect of the invention is the control
elements which make the transcription of the proinsulin
gene responsive to the levels of extracellular glucose.
35   Since the enzymes GLUT-2 and glucokinase are believed
essential for glucose "sensing", hepatocytes, which
produce the enzymes, make a good candidate for a
replacement beta cell. Although, the mechanism of

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"sensing" is not known, Applicants postulated that in
addition to GLUT-2 and glucokinase, the rest of the
"sensing" machinery would be intact in hepatocytes,
including formation of any substances mediating gene
5   expression at the transcriptional level.

     To test this hypothesis, Applicants utilized
varying combinations of different glucose inducible
regulatory elements to determine the time course of
insulin mRNA upregulation. As used herein, a glucose
10   inducible regulatory element (GIRE) contains two
perfect CACGTG motifs separated by five base pairs.

     In the first set of experiments, the first module
utilized, designated sequence C, contained four perfect
CACGTG motifs. The second module, designated sequence
15   F (SEQ ID NO: 5), was based on the module found in
fatty acid synthetase. The first 21 bp constitute a
perfect match of glucose inducible module containing
two perfect CACGTG motifs but then an 11 bp long
segment (10-20 bp of the oligonucleotide), CACGTGGGCGC,
20   is repeated a plurality of times (at least twice, and
preferably three to six times), creating a series of
glucose inducible regulatory elements joined head to
tail.

     These constructs were inserted upstream of the 5′
25   untranslated region of human preproinsulin gene and
then cloned into an adenovirus vector which was used to
transfect hepatocytes. Two sets of experiments were
conducted. First, the sensitivity of regulation in
response to physiological levels of glucose was
30   analyzed by Northern blotting. Strong induction of
insulin mRNA synthesis seen at glucose concentrations
of greater than 5.5 mM. The gels show that both
glucose regulatory modules, as described, are
functional to about the same degree, although the AdFAM
35   construct using the F module appears to be somewhat
more responsive. Next, the temporal response of the
construct containing module C to glucose was tested.
This experiment demonstrated that upregulation of

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insulin mRNA occurs within 30 minutes of exposure to
supraphysiological levels of glucose. Together, these
experiments demonstrate that GIREs provide
transcriptional regulation of insulin mRNA synthesis in
5   hepatocytes. Furthermore, the constructs respond
within a time period necessary for use in treating
diabetes, in contrast to Shih et al., J. Biol. Chem.,
269: 9380 (1994, which measures total accumulation of
CAT at 48 hours.

10   Further analysis of the construct described above
indicated that the rat albumin promoter and insulin 5'-
untranslated region contained an approximately six base
pair inverted repeat. This inverted repeat has the
potential to form a loop in the insulin mRNA, which
15   inhibits post-transcriptional processing and
translation.

To address this the problem, two chimeric gene
cassettes including the 5'-untranslated region of rat
albumin (bases 153-188 of the sequence published as
20   GenBank Accession No. M16825, incorporated herein by
reference) fused to the cDNA for human preproinsulin
were amplified by PCR as described in Example 3. The
insulin cDNA was previously modified to be cleavable by
furin as described above. The constructs were
25   designated M2A and M2B. These constructs differed only
in that M2A terminated at the end of the human
preproinsulin cDNA at base 382 while M2B includes 18
bases of the 3'-untranslated region of human
preproinsulin. These promoterless constructs were then
30   cloned into plasmid pcDNA3, which contains a CMV
promoter. Expression from these plasmids was assayed
in Cos7 cells. The data (presented in Fig. 11 and 12)
demonstrates that these constructs are capable causing
the production and secretion of insulin.

35   Applicants also compared insulin production from
constructs containing either 1 (SEQ ID NO: 8), 2 (SEQ
ID NO: 9) or three (SEQ ID NO: 10) GIREs incombination
with the rat albumin promoter, rat albumin 5'

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untranslated region and preproinsulin sequence M2B.
The complete sequences of these constructs, which were
inserted into adenovirus vectors, are listed as SEQ ID
NO: 11 (containing 1 GIRE), SEQ ID NO: 12 (containing 2
5   GIREs), and SEQ ID NO: 13 (containing 3 GIREs).
Insulin production in hepatocytes transfected with
these vectors was analyzed. Hepatocytes transfected
with the vector containing 1 GIRE demonstrated only low
levels of insulin production. Hepatocytes transfected
10 with the vectors containing either 2 or 3 GIREs
demonstrated high levels of insulin production in
response to physiologically relevant levels of
hyperglycemia. It is expected that the baseline
production of insulin will plateau with increasing
15 numbers of GIREs. Furthermore, the construct
containing 3 GIREs was more responsive to 10mM glucose
than the construct containing 2 GIREs.

Shih, et al., supra, discloses different
combinations of perfect and imperfect motifs in
20 combination with a L-PK TATA box, driving a CAT gene.
The first construct consists of the wild type L-PK
promoter driving CAT. The GIRE in this promoter
consists of two imperfect motifs, having the sequences
CACGGG and CCCGTG. Each of these motifs contains a one
25 base pair mismatch. The second construct consists of a
S14 wild type GIRE in combination with the L-PK TATA
box, driving a CAT gene. The GIRE in this construct
consists of one perfect motif with the sequence CACGTG
and one imperfect motif, having a two base pair
30 mismatch, with the sequence CACGCG. The third
construct consists of two S14 wild type GIREs, combined
with the L-PK TATA box, driving a CAT gene. This
construct has two GIREs, each having a perfect CACGTG
motif and an imperfect motif, having a two base pair
35 mismatch, with the sequence CACGCG.

CAT expression from these constructs was measured
after the cells were cultured in 5.5 or 27.5 mM
glucose for 48 hours. CAT activity in cell extracts

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was expressed as a percentage conversion of
chloramphenicol to its acetylated form. The L-PK
construct demonstrated high expression of CAT after 48
hours in 27.5 mM glucose. Note that the motifs in this
5   construct contained a one base pair mismatch. The
construct containing one S14 wild type GIRE consisting
of a perfect CACGTG motif and an imperfect motif with a
two base pair mismatch displayed only low levels of CAT
expression. The vector consisting of two S14 wild
10   type GIREs, the GIREs consisting of one perfect motif
with the sequence of CACGTG and one motif with a two
base pair mismatch, also exhibited high levels of CAT
expression.
       The reporter system used for assaying glucose
15   inducible regulation of transcription by GIREs in *Shih
et al.* measured CAT activity after cells transfected
with the vectors had been cultured with glucose at
concentrations of 5.5 or 27.5 mM for 48 hours.
Therefore, this gene expression assay measured the
20   accumulation of the CAT protein in cell extracts, and
did not measure mRNA synthesis directly. Therefore, the
accumulation of CAT activity in the cell extracts is
consistent with the slow up-regulation of transcription
from the CAT gene. The Applicants' data demonstrates
25   that constructs containing two GIREs expressed high
levels of insulin mRNA after only 30 minutes of
exposure to glucose.
       The regulatory module for transcriptional
responsiveness to glucose in the present invention is a
30   synthetic oligonucleotide having at least two GIREs,
each GIRE containing the two operative regulatory motif
segments CACGTG flanking a nucleotide linker segment,
conveniently of the sequence GGCGC. Preferably, the
regulatory module contains from 2 to 8 GIREs, and most
35   preferably the module contains from 3 to 5 GIREs.
Additional GIREs are not believed to effect futher
upregulation as a plateau of biding efficiency is to be
expected. However, additional GIREs above 5 are not

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expected to be detrimental to regulating function. The
ends of the double stranded oligonucleotide module are
synthesized to include half site restriction sequences
to facilitate cloning. For example, for cloning in the
5   preferred defective viral vector described hereinafter,
each sense oligonucleotide starts with a Not I half
site on the 5' end, and each antisense oligonucleotide
includes an Eco RI half site on the 5' end.

A functional cassette includes the proinsulin
10   gene, the glucose regulatory module, and a promoter.
The promoter is preferably a relatively strong
constitutive promoter normally operative only in the
host cell of choice, and responsive to the regulatory
module located on its 5' end. In the cassette devised
15   herein, the rat albumin promoter happened to be
selected, although many other candidates are known in
the art. Using the published sequence, (Heard, et.
al., "Determinants of Rat Albumin Promoter Tissue
Specificity Analyzed by an Improved Transient
20   Expression System", Mol. Cell. Biol., 7: 2425-2434
(1987), incorporated herein by reference) PCR primers
were synthesized containing Eco RI and Kpn I
restriction sites, as indicated in Table 1 and
designated SEQ ID NOS: TA420 (6) and TA421 (7).
25   Nucleotides 1-184 were thereupon amplified. The
amplified rat albumin promoter fragment was purified,
and cut with restriction enzymes Kpn I and Eco RI.
After cloning into pBlueScript, the sequence was
verified by conventional sequencing techniques.
30   Preferably the PCR amplification is carried out
utilizing the pfu polymerase obtainable from
Stratagene, which has a significantly lower error rate
than other polymerases.

The 5' untranslated region of insulin is replaced
35   with the 5'-untranslated region of rat albumin fused in
correct reading frame sequence (in native orientation)
with substantially full-length human preproinsulin
cDNA. Those skilled in the art will understand that

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other 5'-untranslated regions may be substituted for
the rat albumin 5'-untranslated region.   It is
important that adequate spacing (about 25 base pairs)
be provided between the GIRE module and the
5   transcription start site and that the 5' untranslated
region contains as little secondary structure as
possible so that there is proper binding to and
processing by ribosomes.   The use of the 5'-
untranslated region of rat albumin may therefore be
10   seen as a guide to the construction of other cassettes.
Substantially full-length human preproinsulin cDNA
means the full-length human preproinsulin cDNA as well
as other truncated or substituted preproinsulin cDNAs
and genomic DNAs, the transcription and translation of
15   which produce insulin having biological activity.
        The cassette comprising, 5' to 3', a glucose
regulatory response module, a transcriptional promoter
whose level of transcription can be further increased
by the glucose response module, and the structural gene
20   for proinsulin genetically modified to be cleavable by
a host cell endogenous endopeptidase is spliced
together and ligated by conventional techniques.   The
molecular ends of the polynucleotide cassette
preferably have single stranded sequences defining the
25   half restriction site corresponding to complementary
half sites on the vector into which it is to be
inserted.
        The best available vector is a helper-free
replication defective plasmid derived from the
30   adenovirus genome, and described in Newgard, et al.,
"Glucose-Regulated Insulin Secretion," in Molecular
Biology of Diabetes, eds. Draznin, et al., Humana
Press: 1992.   Figures 2-5 diagram the genetic
components and construction of the vector containing
35   the gene cassette.   The advantages of this vector
include the absence of helper virus, thus preventing
propagation of virus and high efficiency of infectivity
of host cells.   It has the disadvantage of being

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diluted out of replicating cells, since adenovirus
integrates the host cell genome with very low
efficiency. Other transducing systems useful in the
present invention include certain integrating

5   retroviral systems and another helper-free recombinant
adenoviral system disclosed in U.S. Patent No.
5,436,146.

Another advantage of the viral-derived vectors is
that delivery to target cells in the intact animal does

10   not require excision of tissue, invitro infection, and
reimplantation. Nothing however, would preclude the
use of an allogenic source of cells under conditions of
immunosuppression. A purified viral stock (2-40
infective units per target cell) may be injected into

15   the hepatic portal vein, with efficient infective rates
obtainable as viral particles penetrate the hepatic
capillary beds and come into contact with the
hepatocytes. In this way, replacement beta sites are
generated insitu without disturbing the normal cellular

20   architecture. Further advantages of the present
invention will become apparent from the Examples which
follow. Alternatively, hepatocytes may be transfected
ex vivo and then transplanted into the human.

It will be apparent that any structural gene for

25   which glucose modulated control is desired, may be
inserted into the gene cassette by conventional
recombinant techniques and expressed in an appropriate
host cell. For proteins not requiring further
processing, the fucin enzyme of hepatocytes is, of

30   course, superfluous. A number of metabolic diseases
for which the present invention has therapeutic value
in restoring a glucose response mediated protein
function can be identified.

35   EXAMPLE 1
Generation and Cloning of Insulin Gene Cassettes in the
plasmid pACdeltaCMV.

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The plasmid pACCMV.pLpA (Fig. 2), used as a vector
for generation of replication defective recombinant
adenovirus containing genes of interest, was cut to
completion with the restriction enzyme Sal I and
5   partially with the enzyme Not I. The 8.3 kb piece of
KNA, lacking CMV promoter, was gel purified and used as
vector for inserting insulin gene cassettes.

The oligonucleotide pair corresponding to one of
the GIREs was mixed with gel purified Eco RI - Kpn I
10   albumin promoter and Kpn I - Sal I InsM1 DNA fragments,
the mixture was ligated with the above described
plasmid vector pACΔCMV. A combination of a Glucose
Regulatory Response Modules C or F (Figure 1), albumin
promoter and the mutant insulin cDNA, produced a total
15   of two constructs. Integrity of both the constructs
was confirmed by sequence analysis. Each of the two
constructs was cotransfected with the plasmid pJM17 in
the host 293 cell line, as described, to generate
recombinant replication-defective adenovirus
20   constructs, namely Ad.CAM1 and Ad.FAM1 (see Figure 5).

EXAMPLE 2
Expression of Insulin in Hepatocytes at various glucose
concentrations.

25

Rat hepatocytes were prepared by *in situ* perfusion
of 0.5 mg/ml collagenase in supplemented balanced
Hank's solution as described (Kreamer et. al. (1986) In
Vitro 22, 201-211). The viability of isolated
30   hepatocytes was 90% or better.

Six collagen coated 60 mm plates, each containing
1×10^6 hepatocytes, were transfected with 5×10^7
pfu/plate. The transfected hepatocytes were exposed to
three concentrations of glucose, 3.3 mM, 5.6 mM and
35   27.5 mM, in RPMI supplemented with 10% fetal calf
serum, 30 μg/ml proline, 5 μg/ml insulin, 5 μg/ml
transferrin and 5 μg/ml selenium. After 36 h, one of
the two plates at each of the tested glucose

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concentrations, was used to prepare RNA and the other
plated was used to check the viability of the
hepatocytes. Hepatocyte viability at all the tested
concentrations of glucose were no more than 10%

5   different. Ten microgram of RNA from each sample was
electrophoretically resolved on a formaldehyde-2%
agarose gel, the RNA transferred to a Nylon membrane,
UV-crosslinked and hybridized with digoxygenin-labeled
insulin cRNA. Detection of the membrane-bound probe

10   was performed by chemiluminescence, results recorded as
multiple exposures on X-ray films for various lengths
of time and quantitated by digital image analysis.
          Referring to figures 6 and 7, Northern analysis
reveals that RNA migrating at the position of

15   polynucleotides of approximately 1.35 kb, corresponding
to the predicted size of the proinsulin transcript, is
evident only when transfected hepatocytes are cultured
in the presence of 27.5 mM glucose. Importantly, no
induction of the proinsulin gene over background is

20   indicated at 3.3 or 5.5 mM glucose. Unlike other
attempts at constructing an artificial inducible
insulin-producing replacement cell, in which induction
occurs at subphysiological levels of glucose, the
present transfected hepatocytes show a response only in

25   physiological or supraphysiological range. Strong
induction is seen at glucose concentrations of greater
than 5.5 mM. A strong response is apparent at 10mM.
The gels show that both glucose regulatory modules, as
described, are functional to about the same degree,

30   although the AdFAM construct using the F module appears
to be somewhat more responsive. As a control, the
Ad.CMP-Ins in which the gene of interest (proinsulin or
beta-galactosidase) is under control of the
constitutive CMV promoter, generates RNA of the

35   distinctive size without regard to glucose
concentration and is used to quantitate the amount of
insulin mRNA (Figures 6, 7 and 8).

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Quantitation by phosphoimaging is summarized in
Table 2. The results show only a slight difference in
relative expression between 3.3 and 5.6 mM glucose, in
contrast to a 3.06 value at 27.5 mM. Figure 9 shows the
5   gel normalizing rRNA and mRNA to arrive at the 3.06
value.

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Table 1

Oligo	Sequence (5'-3')
TA403	GAG GGG TCC CGG CAG AAG CGT GGC A
TA404	ACG CTT CTG CCG GGA CCC CTC CAG G
TA413	__Sal 1__ .CGG *AGT CGA CCA* TCT CTC TCG GTG CAG GAG GCG G
TA420	__Eco R1__ GGA *ATT CTC* TAG AGG GAT TTA GTT AAA CAA CTT
TA421	__Kpn 1__ GGG *GTA CCA* GAG GCA GTG GGT TGA CAG GT
TA423	__Kpn 1__ GGG *GTA CCA* TCA GAA GAG GCC ATC AAG CA

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Table 2

Quantitation of Insulin mRNA in Hepatocytes

Glucose (mM)	hIns mRNA	18s RNA	Normalized hIns mRNA (hIns mRNA/18s RNA)	hIns mRNA (Relative* Expression)
3.3	81074	56	787	0.91
5.6	51039	33	865	1.00
27.5	201056	40	2645	3.06

*For the sake of comparison, the normalized amount of insulin mRNA expressed at euglycemic level (5.6 mM glucose) is arbitrarily assumed to be one.

EXAMPLE 3:
CLONING OF INSULIN CONSTRUCT WITH RAT ALBUMIN 5'
UNTRANSLATED REGION

The chimeric insulin genes of Example 1 were further modified by eliminating the 5' untranslated region of human insulin mRNA and replacing that region with the 5' untranslated region of rat albumin (bases 153-188 as published in GenBank Accession No. M16825, herein incorporated by reference). This modification was accomplished by PCR amplification of hInsM2 using three oligonucleotide primers (sequences listed in Table 3). The oligonucleotide TA455 (5'-3') comprises a sequence corresponding to the restriction enzyme Kpn I recognition site, bases 153-188 of the albumin promoter corresponding to the 5'-untranslated region of albumin and a sequence corresponding to human preproinsulin mRNA (starting with the first amino

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acid). The reverse (antisense) oligonucleotide TA452
terminates at the end (base 392) of the translated
sequence of the human insulin cDNA sequence. The
reverse (antisense) oligonucleotide TA454 corresponds
5   to the 3' region of human preproinsulin CDNA plus 18
bases of the 3' untranslated region of human insulin
(bases 393-410). Both of these reverse
oligonucleotides also included a Sal I restriction site
to facilitate cloning of the generated products into
10   the small pACCMV based plasma described in Example 1.
The amplification product of TA455 and TA452 has been
designated as mutant M2A. The amplification product of
TA455 and TA454 has been designated as the mutant M2B.

       After amplification, the modified chimeric insulin
15   genes were then cloned without their promoter sequences
into the commercial plasmids vector pcDNA3 containing
the CMV promoter. The steps involved in cloning and
expression are schematically depicted in Figure 10.
Since pcDNA3 does not have a unique Sal I site, the
20   orientation of insertion was controlled by using Kpn I
and EcoRV. The insulin gene mutants M2A and M2B were
digested with Kpn I and separately ligated into the
pcDNA3 vector. After transformation of E. coli using
the ligated DNA, plasmids containing the two insulin
25   mutants were prepared and used to transfect Cos7 cells.

EXAMPLE 4:
SECRETION OF INSULIN FROM COS 7 CELLS TRANSFECTED WITH
30   INSULIN CONSTRUCTS CONTAINING THE 5'-UNTRANSLATED
REGION OF RAT ALBUMIN

       Cos7 cells were used to test the ability of the
constructs described in Example 3 to synthesize and
35   secrete insulin. The TRANS IT™ transfection reagent
(Pan Vera Corp., Madison, Wisconsin) was used to
transfect the Cos7 cells with plasmids containing the
M2A and M2B mutants for transient expression. A four

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hour period of incubation for transfection was followed
by an overnight incubation of the cells and fresh DMEM
supplemented with 10% fetal calf serum. The medium was
then changed and the plates incubated for two days in
medium containing either 10% or 5% fetal calf serum.
The medium and the cells were harvested separately, and
the cells lysed in Tris-buffered saline (pH 7.6)
containing 1% NP-40 and protease inhibitors (trypsin
inhibitor and PMSF). Both the medium and cell lysate
were analyzed for the presence of insulin by antigen
capture ELISA.

The preliminary results demonstrate that both
clones are capable of causing insulin synthesis and
secretion. M2B demonstrates a tendency towards higher
insulin production (see Figures 11 and 12). It is also
apparent that the majority, if not all, of the
synthesized insulin is secreted (Figure 12). Control
cells transfected with the vector pcDNA 3 with no
insert did not produce insulin. The measured levels of
insulin in these controls was not significantly
different that the baseline of the insulin standard
curve.

TABLE 3

Oligonucleotides Used for Amplification
of Insulin Mutants M2A and M2B

Sequence Name	Sequence Length	Sequence
TA455	64	*ggg gTA CCg* ACC ACC TTT CCT gTC AAC CCA CTg CCT CTg gCA CAA Tgg CCC TgT ggA TgC gCC T
TA452	30	*Cgg AgT CgA* CCT AgT TgC AgT AgT TCT CCA
TA454	30	*Cgg AgT CgA* CgC TgC CTg CAg gCT gCg TCT

Example 5:

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Quantification of glucose-dependent insulin release
from hepatocytes transfected with adenovirus constructs
containing insulin and 1, 2 or 3 GIREs.

5   The examples above included data showing that
glucose-inducible transcription of hIns in rat
hepatocytes is achieved via a genetic construct with
hIns cDNA under control of a chimeric serum albumin
promoter containing glucose-inducible regulatory
10   elements (GIREs). The information regarding the
ability of hIns mRNA to produce and secrete insulin
protein was also provided using COS7 cells.

         To study expression in hepatocytes, three chimeric
human insulin constructs in adenovirus, containing 1, 2
15   or 3 GIRE units were constructed. Followed by the
specific number of GIRE(s), each construct contains
albumin promoter and human insulin cDNA (including the
two sets of mutations to aid furin mediated processing
of proinsulin to insulin).

20   The general strategy of assembling the hIns
constructs was essentially the same as described above.
The sense and antisense oligonucleotides corresponding
to 1 or 2 GIREs were chemically synthesized. Each set
of oligonucleotide pairs was designed such that when
25   annealed together, the double stranded DNA contained
the sticky ends for the restriction enzymes Not I and
EcoR I on 5' and 3'-ends, respectively. An additional
pair of oligonucleotides corresponding to one GIRE was
made to contain EcoR I and XbaI sites on the 5' and 3'-
30   ends, respectively. The original sense oligonucleotide
used for amplification of rat albumin promoter contains
EcoR I site followed by Xba I site. The last pair of
oligonucleotide (1GIRE) was inserted into the construct
containing 2 GIREs using EcoR I-Xba I, thus giving rise
35   to the construct with 3 GIREs.

         The rat albumin promoter sequence described
earlier was extended by PCR to include the entire 5'-
untranslated region of rat albumin. The human insulin

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cDNA containing two sets of mutations, corresponding to the B/C and C/A junction of proinsulin, from constructs previously described was modified to eliminate the 5'-untranslated region arising from hIns cDNA by PCR. The
5 two fragments of DNA were joined together by overlap extension in a PCR reaction. The product of this reaction contained (5'->3') albumin promoter, 5'-UTR of albumin and translated sequence of hIns modified to be compatible with furin. This DNA fragment containing
10 EcoR I and SalI restriction enzyme sites on 5' and 3'-ends, respectively, was digested with the two enzymes, mixed with the annealed pair of oligonucleotides corresponding to 1 or 2 GIREs, described above, and ligated into the plasmid vector used to produce
15 adenovirus (pACCMV from which CMV promoter has been deleted).

Applicants next determined whether the glucose-induced upregulation of hIns mRNA is accompanied by a corresponding glucose-induced secretion of hIns protein
20 and if the extent of glucose-induced insulin secretion varies depending on the number of GIRE units used per construct. Plasmids corresponding to 1, 2 or 3 GIREs were individually mixed with the plasmid pJM17 and the mixture of the two plasmid DNA cotransfected into HEK
25 293 cells to generate recombinant replication-defective adenovirus (as described earlier). Each recombinant hIns construct was sequenced to ascertain that no errors were introduced by PCR or during subsequent cloning procedures. Freshly prepared hepatocytes were
30 plated in collagen coated 30mm plates and transfected (MOI=4) with adenovirus containing insulin construct with 1 (Ad1SAM2B), 2 (Ad2SAM2B) or 3 (Ad3SAM2B) GIREs, as indicated. The culture medium was changed 16h post-transfection, with medium containing 2.5, 5.6, 10 or
35 27.5 mM glucose, as indicated. The insulin present in medium was assayed by antigen capture ELISA 32h later (the numbers shown in Table 4 represent ng Insulin/ml +

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SD). The results are presented in Figs. 15, 16 and 17 and in Table 4.

A glucose-dependent increase in the secreted insulin from transduced hepatocytes is clearly seen
5   when the gene construct contains either 2 (Fig. 16) or 3 (Fig. 17) GIREs. However, in case of the construct having 1 (Fig. 15) GIRE, the overall insulin secretion as well as glucose induction is minimal. Furthermore, the construct containing 3 GIREs, in addition to a
10   higher maximal induction at 27.5 mM glucose (approximately 9 fold increase over the level at 2.5 or 5.6 mM glucose as opposed to 6.5 fold increase in the case of construct with 2 GIREs under identical conditions), also shows >2 fold increase in insulin
15   secretion at 10 mM glucose.

TABLE 4

Insulin secreted from Hepatocytes transfected with
20   vectors containing 1, 2, or 3 GIREs

Glucose, mM	Ad1SAM2B	Ad2SAM2B	Ad3SAM2B
2.5	4.97 $\pm$ 1.09	9.09 $\pm$ 2.27	9.98 $\pm$ 1.37
5.6	5.12 $\pm$ 1.26	10.72 $\pm$ 2.30	10.80 $\pm$ 1.57
10.0	3.2 $\pm$ 1.8	13.2 $\pm$ 2.4	22.8 $\pm$ 4.1
27.5	12.75 $\pm$ 2.05	67.16 $\pm$ 10.24	90.33 $\pm$ 19.80

30   Example 6.

Temporal response of GIRE constructs to
physiological levels of glucose.

Collagen coated 60 mm plates or dishes, each
35   containing 1 X 10^{6} hepatocytes, were transfected with 3.5 X 10^{6} pfu/plate of the test adenovirus containing the insulin gene and two GIREs. Control plates received either no virus, or virus encoding bacterial B-galactosidase. Hepatocytes were then exposed for 16h

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to 5.6 mM glucose in RMPI supplemented with 10% fetal
calf serum, 30 μg/ml proline, 5 μg/ml insulin, 5 μg/ml
transferrin and 5 μg/ml selenium, at 37 degrees
Centigrade. The plates containing transfected cells
5   were then divided into two groups, one group receiving
fresh medium containing 5.6 mM glucose, the second
group receiving fresh medium with 27.5 mM glucose.
From each of these two groups, individual plates were
removed after 30 min, 1h, 2h, 4h, 8h, and 16h, the
10   medium decanted, and the cells frozen in liquid
nitrogen. Total RNA was extracted and analyzed for
hIns mRNA by Northern blotting.

The Northern blot in Figure 13 demonstrates that
after exposure to 27.5 mM glucose, hIns mRNA was
15   detectable at the first time point of 30 min and
increased thereafter in a time-dependent manner. At
the normal glucose level (5.6 mM) the signal was much
lower. Quantitation of the bands revealed that the
upregulation of insulin message observed at 27.5 mM is
20   roughly 10 fold as compared to the 5.6 mM treatment.

These data demonstrate that the vector construct
containing two GIREs initiates transcription in
response to elevated glucose levels in a time-frame
comparable to islet cells. This rapid temporal
25   response to elevated glucose levels confers a level of
control of insulin synthesis in addition to control
mechanisms arising from glucose detection by the GLUT-2
and glucokinase pathways. These data also demonstrate
a precise and correct response to physiological levels
30   of glucose. Insulin mRNA production is not upregulated
in the presence of 5.6 mM glucose, the steady state
concentration of glucose in the bloodstream. Insulin
mRNA synthesis is stimulated by elevated (above 5.6 mM)
levels of glucose.

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Example 7.

   Insulin synthesis at physiological ranges by
hepatocytes transfected with construct containing 2
GIREs.

5

      Freshly prepared rat hepatocytes were transfected
with two different adenovirus constructs containing the
M1 mutated insulin gene: AdSAM1 (containing the rat
Albumin promoter modified to contain 2 GIREs) and
10   AdCMVInsM1 (containing the constitutive and highly
active CMV promoter). Hepatocytes transfected with
AdCMV.β-Gal and untransfected hepatocytes were used as
controls. Four plates of hepatocytes were transfected
with each adenovirus preparation; two plates were
15   exposed to the low (3.3 mM) glucose and the other two
plates to the high (27.5 mM) glucose concentration.
After 36h, hepatocytes were exposed for 16h to 5.6 mM
or 27.5 mM glucose in RMPI supplemented with 2 mg/ml
bovine serum albumin with leucine omitted, 30 μg/ml
20   proline, 5 μg/ml insulin, 5 μg/ml transferrin and 5
μg/ml selenium, at 37 degrees Centigrade.
      Following a 6h leucine depletion, a 2 ml aliquot
of the low or high glucose containing defined medium
was added to appropriate plates. For each adenovirus
25   used, one plate for each glucose concentration received
0.2 mCi^{3}H-leucine (500 Ci/mmole). The remaining plate
received the equivalent amount of unlabelled leucine
and at the end of all incubations it was used for
viability determination.
30   The leucine incorporation was carried out for 16h,
followed by a 4h chase with unlabeled leucine. The
culture medium was aspirated, cell debris removed, and
the supernatant used for analysis of secreted products.
The cells on each plate were lysed with 0.8 ml solution
35   containing 20 mM Tris-HCL buffer at pH 7.6, 2 mM EDTA,
5 μg/ml trypsin inhibitor, 50 μM phenylmethane
sulphonyl fluoride (PMSF) and 1% Triton-X100. The
lysate was centrifuged at 16,000xg for 10 min in a

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microcentrifuge, the pellet discarded, and the
supernatant solution used for analysis of labeled
intracellular products.

For each analysis of secreted insulin by
5   immunoprecipitation, 0.8 ml of culture supernatant was
used, and for each analysis of intracellular insulin
0.4 ml of cell lysate supernatant was used. Samples
were pre-cleared with *Staph. aureus* in the absence of
specific antibodies, to reduce the non-specific
10   precipitation of labeled proteins during the procedure:
50μl of 10% suspension of formalin-fixed Staphylococcus
cells (from Calbiochem) was added to each tube; tubes
were kept at room temperature for 30 min with
continuous mixing, centrifuged (4 min, 16,000xg) and
15   the supernatant used for further analysis. For
immunoprecipitation of insulin and insulin-related
products, 2.5μl of polyclonal guinea pig anti-human
insulin (from Sigma Chemical Co.) was added to the
supernatant solution from the above step, mixed and
20   kept at room temperature for 45 min, followed by
addition of Staphylococcus cells with 30 min incubation
plus centrifugation exactly as before. Supernatant was
discarded, and the pellet washed 4-5 times with 1 ml of
20 mM Tris-HCl at pH 7.6, 0.15 M NaCl and 0.1% Triton
25   X-100.

The pellet was suspended in 40 μl solution
containing 60 mM Tris-HCl at pH 6.8, 1.2% SDS, 2% β-
mercaptoethanol, heated in a boiling water bath for 4
min, centrifuged, and the supernatant analyzed by
30   polyacrylamide-SDS gel electrophoresis. As an internal
control for sample to sample variation, in a second set
of tubes 2 μl of rabbit anti-rat albumin polyclonal
antiserum was included along with anti-insulin
antiserum, enabling co-precipitation of human insulin
35   and the endogenous rat albumin in the subsequent single
step. Specificity of the immunoprecipitated material
was established by the use of control cells transduced
with an unrelated gene, β-galactosidase, and

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untransfected cells. To further confirm the identity
of the immunoprecipitated material, in a separate set
of tubes unlabeled insulin and rat serum were added to
provide competition with labeled insulin and albumin
5   respectively, and tubes processed simultaneously.

The optimum gel system for resolution of insulin B
and A chains and rat albumin was found to be an
SDS/Tris-Tricine 10-20% linear polyacrylamide gradient
based on the description of Schagger and Jagow (Analyt.
10   Biochem. 166:368-379, 1987). A 15 μl aliquot of SDS-
βME-treated immunoprecipitated material from each
sample was resolved on the gel along with peptide size
markers from BioRad. The gels were fixed, stained,
destained, soaked in "Amplify" solution (Amersham),
15   dried under vacuum, and exposed to X-ray film at -80oC.

Our results (Figure 14) show the presence of an
anti-insulin antibody-binding band in cell extracts of
hepatocytes transduced with both of the insulin cDNA-
containing constructs, AdSAM1 (glucose-inducible) and
20   AdCMV.InsM1 (constitutive). As noted in Methods above,
both constructs contain the M1 insulin cDNA, mutated in
the coding region at the C/A junction. AdSAM1 contains
two glucose-inducible regulatory elements coupled to
the rat albumin promoter, and AdCMV.InsM1 contains the
25   cytomegalovirus immediate/early promoter. When AdSAM1
was used, the insulin band appeared only in the
hepatocytes exposed to high glucose and not the low
glucose. When AdCMV.InsM1 was used, the insulin-
positive band was present in approximately equal
30   amounts regardless of the glucose concentration. It
should be noted that these results were controlled for
possible differences in gel loading and other sources
of sample-to-sample variation, including a difference
in hepatocyte survival after incubation at 3.3 mM
35   versus 27.5 mM glucose (10-15% lower viability at low
glucose), by co-precipitating rat serum albumin and
using it as an internal standard.

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The size of the insulin positive band was determined to be 7,700 Daltons. This differs from the sizes of mature insulin B and A chains and most likely contains C+B peptides of insulin as a result of

5   incomplete processing proinsulin. The size of rat albumin was determined to be 67,000 Daltons, which compares favorably with the known size. The identities of the observed bands as insulin and albumin were confirmed by the fact that the signals were almost

10   completely ablated when excess unlabeled insulin and normal rat serum were included during the immunoprecipitation. A preliminary determination using digital densitometry revealed that intra-cellular insulin protein expression at high glucose was only

15   about 20-fold lower when driven by the chimeric albumin promoter employed in AdSAM1 then when driven by the CMV promoter in AdCMV.InsM1. This is highly encouraging since the CMV promoter is the most active known promoter in most *in vivo* and *ex vivo* mammalian systems.

20   It would not be desirable to express insulin at the levels achieved by the CMV promoter.

These data demonstrate the synthesis of insulin in response to physiological levels of glucose in hepatocytes transfected with a vector containing the

25   proinsulin gene under the control of two GIREs. At 3.3 mM glucose, there is no detectable sythesis of insulin; at 27.5 mM glucose, insulin synthesis is readily detectable. By adding two GIREs to the vector, which provide an additional control mechanism at the

30   transcriptional level, the synthesis of insulin is correctly regulated in the appropriate physiological range. Insulin mRNA and insulin are synthesized at glucose concentrations exceeding 5.6 mM and are not synthesized at glucose concentrations of 5.6 mM. This

35   feature makes vectors containing GIREs uniquely appropriate for treatment of Type 1 diabetes and superior to the prior art vectors.

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TREATMENT OF DIABETES WITH SYNTHETIC BETA CELLS

CLAIMS

5   What is claimed is:

1.   A gene cassette for expression of a proinsulin
gene in a host cell comprising
       a nucleotide sequence coding for a proinsulin
10   cleavable into active insulin and operably linked to a
promoter sequence recognized by an RNA polymerase
contained in said host cell; and
       a glucose responsive regulatory module located 5'
of said promoter comprising four or more CACGTG motifs,
15   said motifs separated by a linker sequence.

2.   The gene cassette of claim 1 further comprising a
nucleotide sequence coding for a 5' untranslated region
of rat albumin located in native orientation between
20   the promoter sequence and the proinsulin sequence.

3.   A vector for transfecting a host cell capable of
transcribing a proinsulin gene contained in said
vector, said vector comprising
25   a gene cassette consisting of a nucleotide
sequence encoding a proinsulin cleavable into active
insulin and operably linked to a promoter transcribable
in said host cell and a regulatory module located 5' of
said promoter, and
30   a replication defective viral genome capable of
expressing the genes required to package said vector
invivo into a viral particle infective for said host
cell.

35   4.   The vector of claim 3 wherein said host cell is a
hepatocyte.

5.   A synthetic glucose responsive regulatory module
comprising

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four or more CACGTG motifs, said CACGTG motifs
separated by a linker segment.

6.   The glucose responsive regulatory module of claim
5 wherein said linker segment joining said CACGTG
motifs is 5'-GGCGC-3'.

7.   A gene cassette for glucose modulated expression
of a structural gene in a host cell comprising
       a nucleotide sequence coding for said structural
gene operably linked to a promoter recognized by an RNA
polymerase contained in said host cell; and
       a glucose responsive regulatory module located 5'
of said promoter comprising four or more CACGTG motifs,
said motifs separated by linker sequence.

8.   A method of treating diabetes comprising
       transfecting cells expressing glucose transporter
2, glucokinase, and an enzyme capable of cleaving a
genetically modified proinsulin to yield a protein
having insulin activity, with a vector comprising
       a gene cassette consisting of a nucleotide
sequence encoding a substantially full length
proinsulin gene operably linked to a promoter
transcribable in said cells, and a regulatory module
located 5' of said promoter,
       a replication defective viral genome capable of
expressing the genes required to package said vector
invivo into a viral particle infective for said cells.

9.   The method of claim 4 wherein said cells are
hepatocytes.

Glucose Inducible Regulatory Elements

C

gC ggC CgC CAg TTC TCA CgT ggT ggC CAC gTg CTT ggg CAC
gCC AgTTCT CAC gTg gTg gCC ACg TgC TTg ggC ACg TTA AC

E

gC ggC CgC TgT CAC gTg ggC gCC ACg Tgg gCg CCA CgT ggg
CgCCAC gTg ggC gCC ACg Tgg gCg CCg TTA AC

FIG. 1
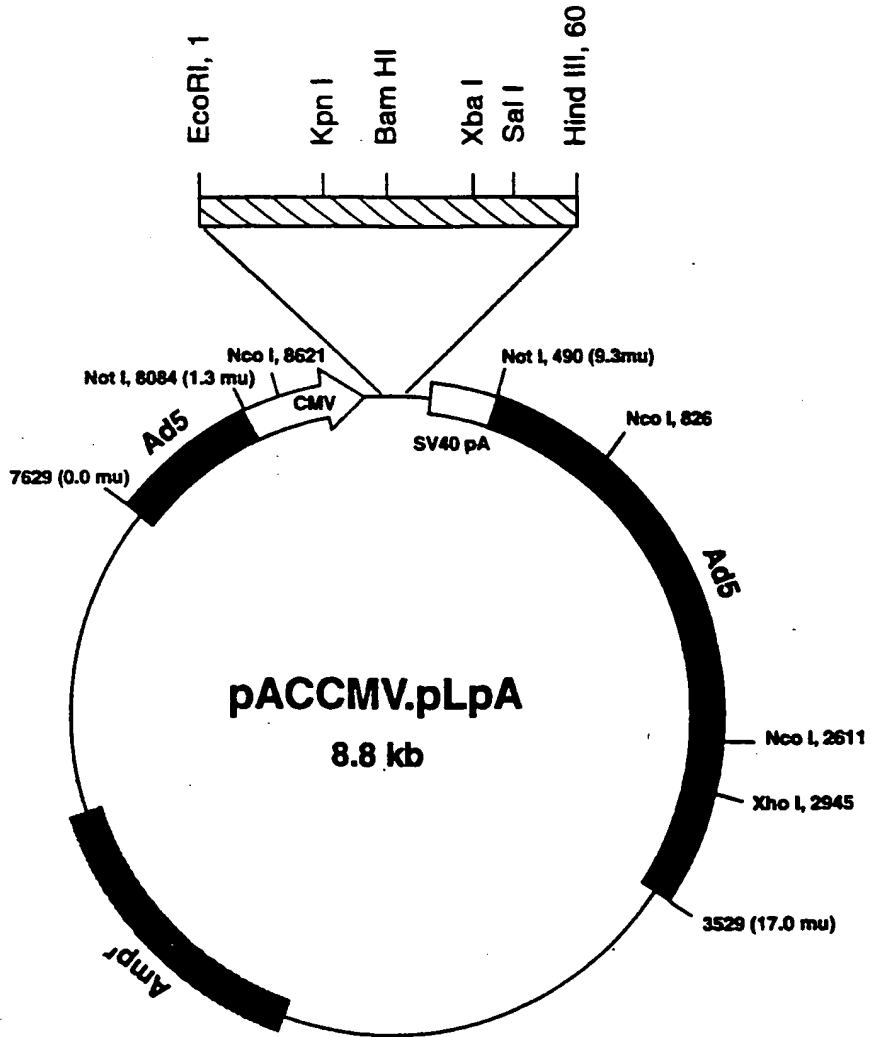
FIG. 2
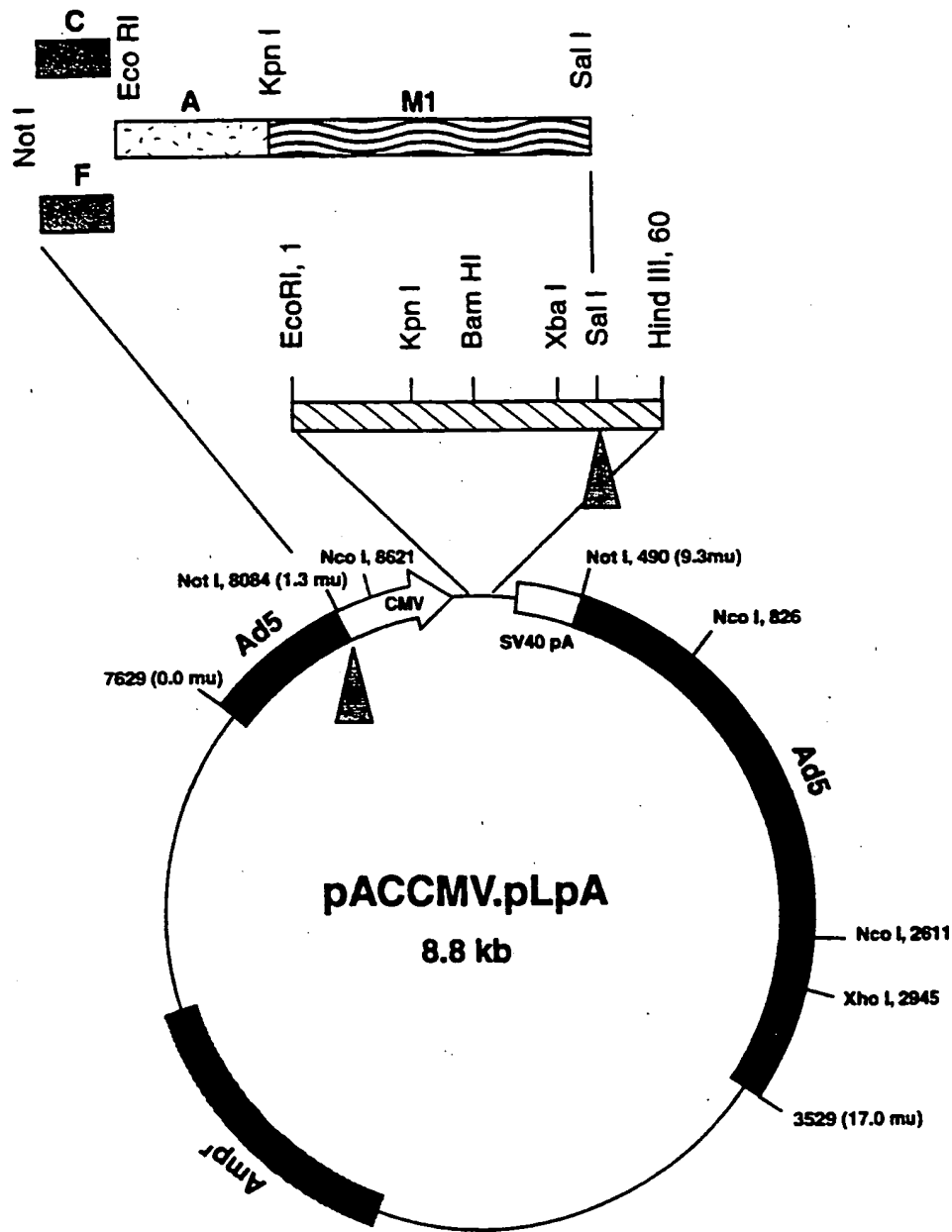
FIG. 3A

Insulin Expression Clones in Adenovirus
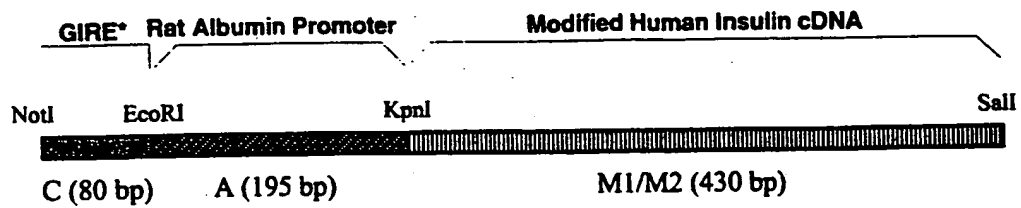
FIG. 3B
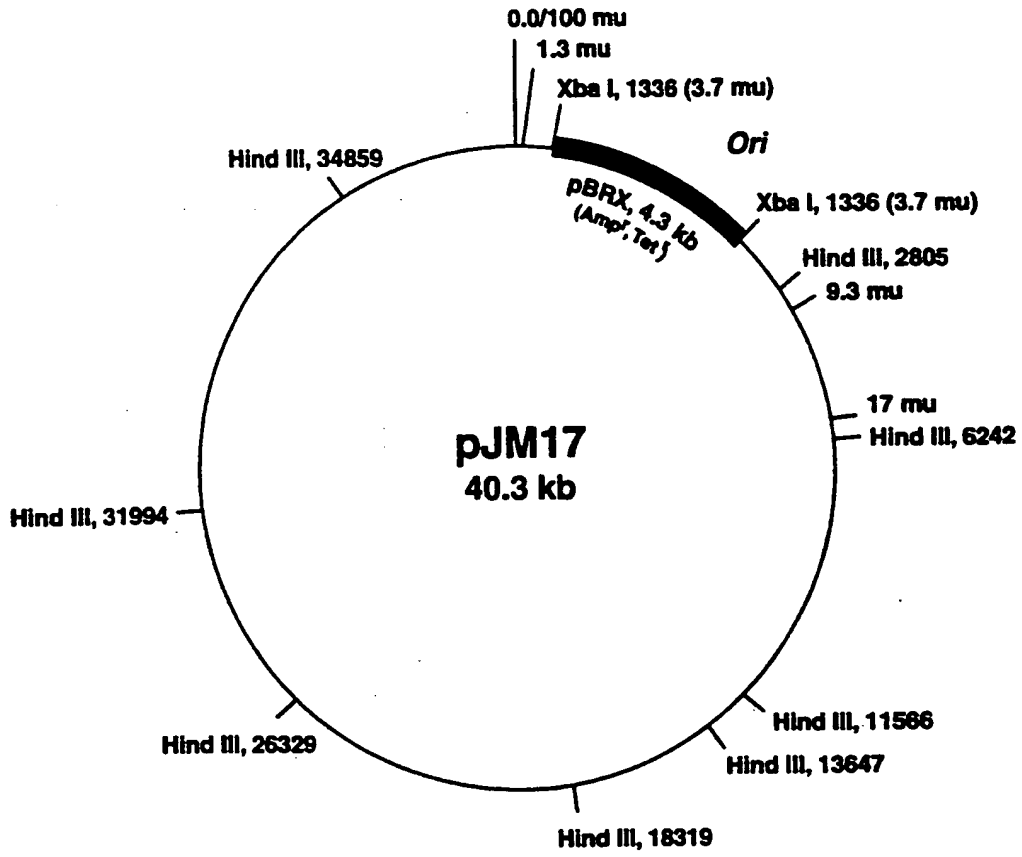
FIG. 4

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FIG. 5

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Northern

FIG. 6

Northern

FIG. 7

Northern

FIG. 8

RNA Blot

Northern

FIG. 9

Human Insulin cDNA mutant M2

5'-UTR   3'-UTR

Kpn I   Sal I

hInsM2/pBSK+

Kpn I + Sal I
digestion

5'-UTR   hInsM2   3'-UTR   5'-UTR   hInsM2   3'-UTR

TA455   TA452   TA455   TA454

PCR   PCR
Amplification   Amplification

Kpn I   SalI   Kpn I   SalI
              InsM2A   InsM2B

pcDNA3   pcDNA3

InsM2A/pcDNA3   InsM2B/pcDNA3
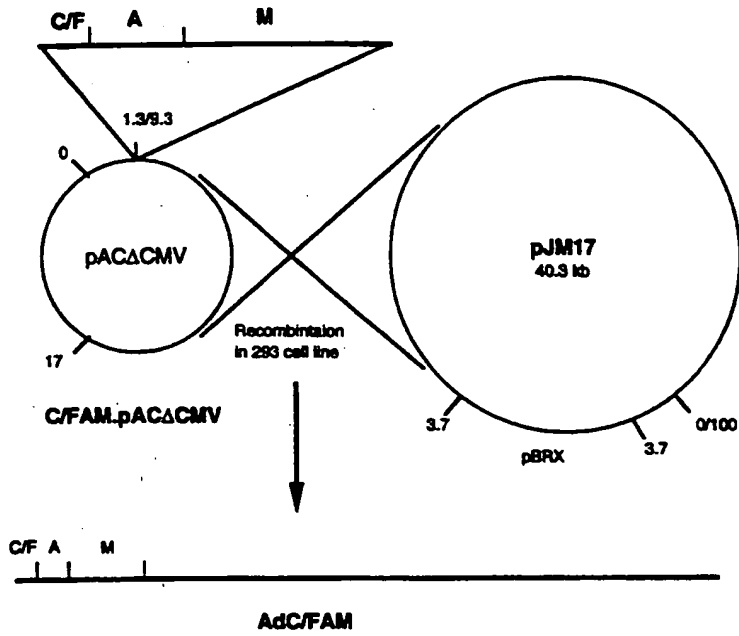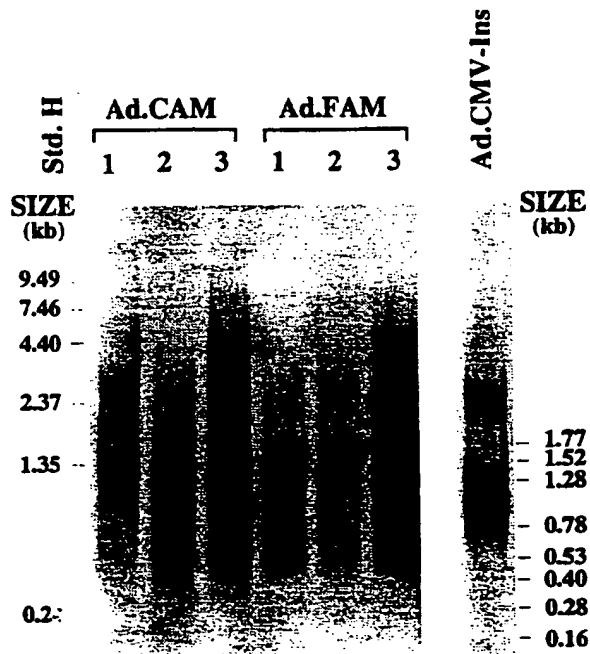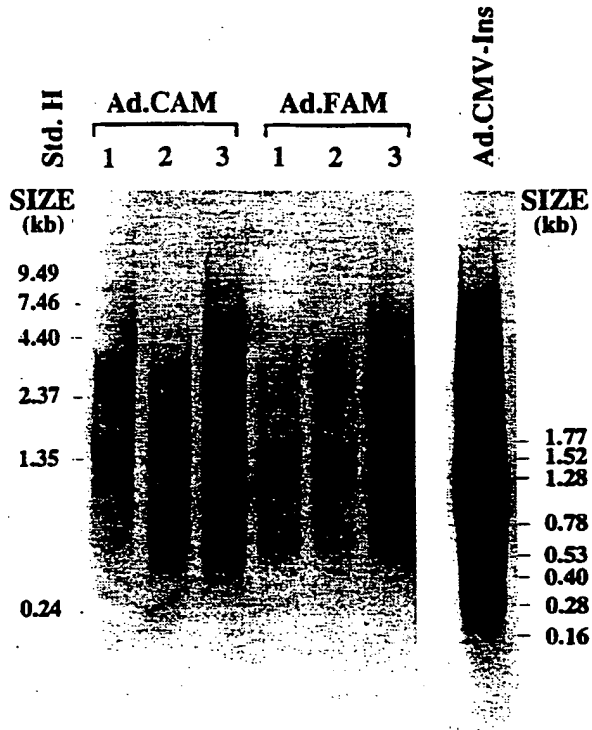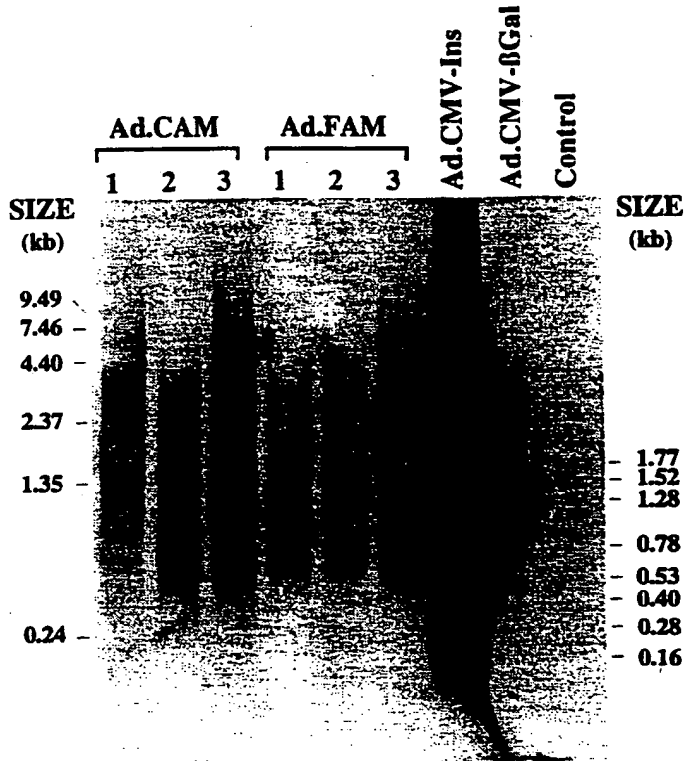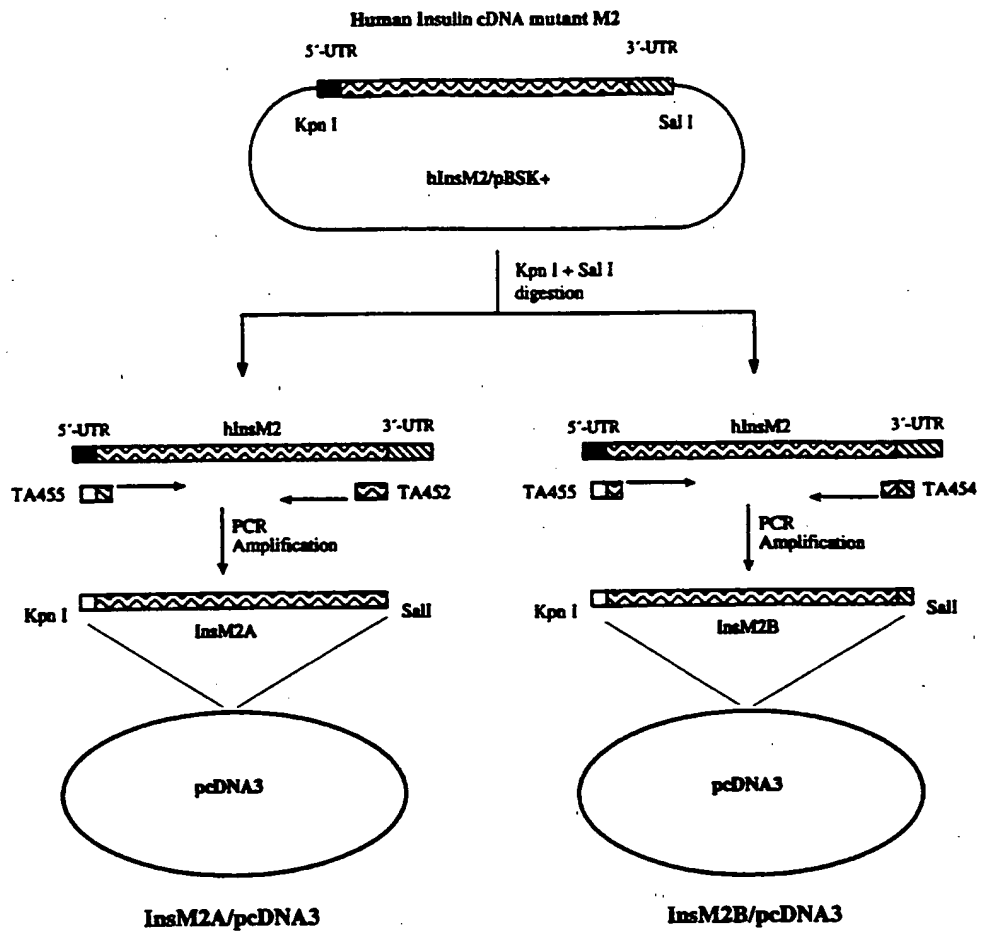
FIG. 10

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Insulin Released from Cos7 Cells Transfected with Recombinant Plasmids
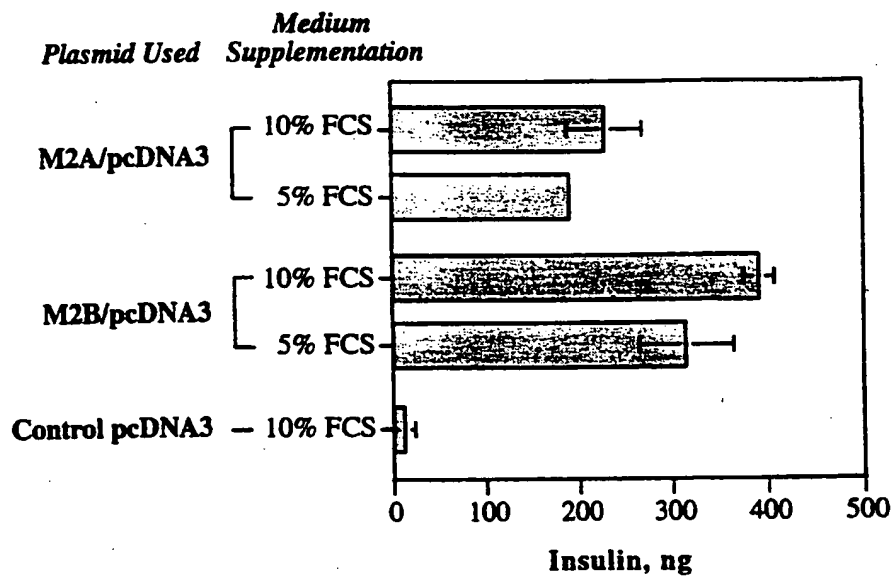
FIG. 11

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**Insulin Released from Cos7 Cells Transfected with Recombinant Plasmids**

Plasmid Used   Localization
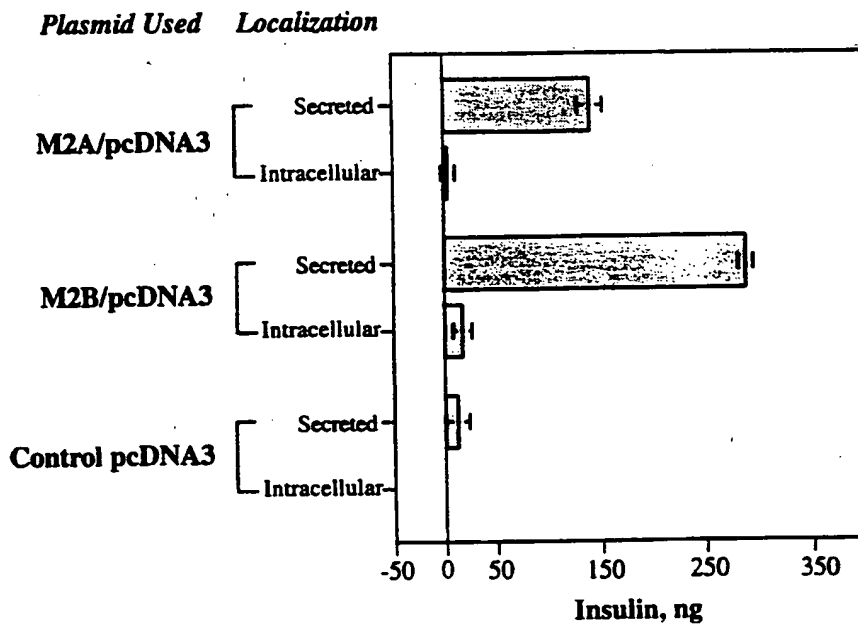
FIG. 12
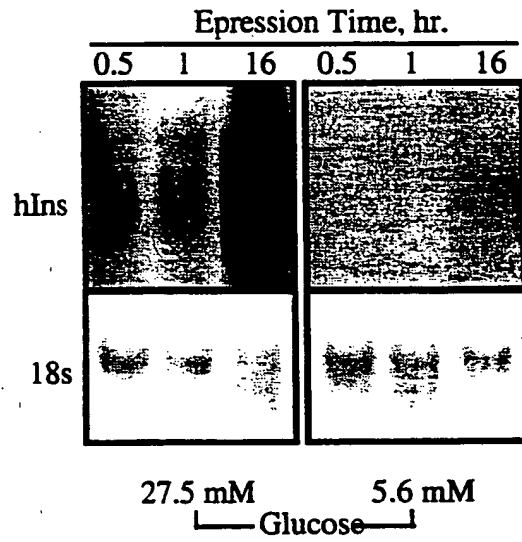
FIG. 13

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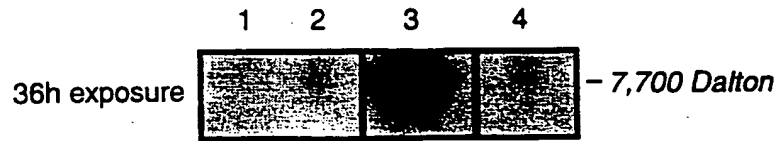
FIG. 14

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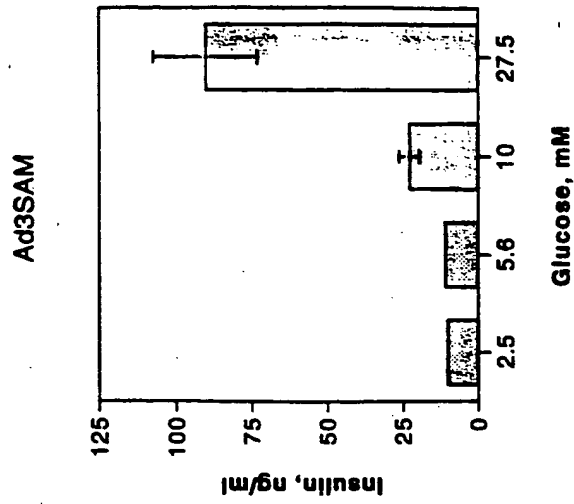
FIG. 17
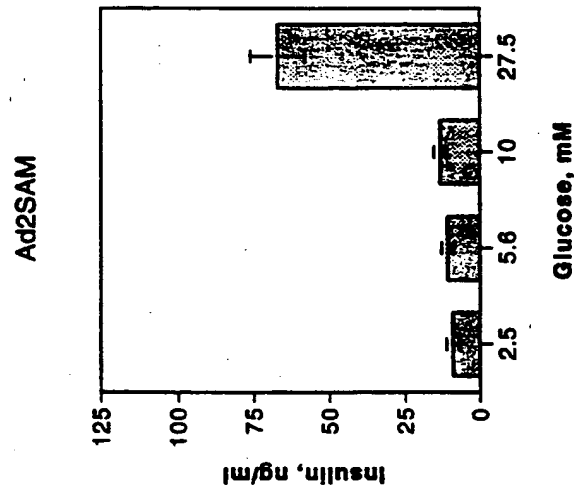
FIG. 16
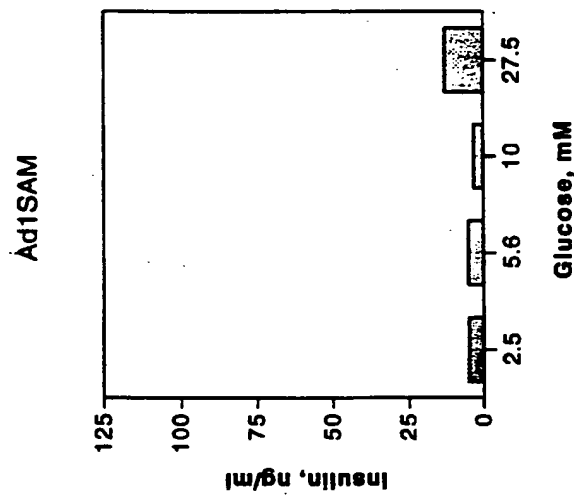
FIG. 15

-1-

SEQUENCE LISTING

<110> Hullett, Debra A.
      Alam, Tausif
      Sollinger, Hans W.
      Theron, Amy

<120> Treatment of Diabetes with Synthetic Beta Cells

<130> CIP Application 96429/9003

<140>
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<150> 08/786625
<151> 1997-01-21

<160> 13

<170> PatentIn Ver. 2.0

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<220>
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SUBSTITUTE SHEET (RULE 26)

-2-

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<220>
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     corresponding to human insulin containing lysine
     and 2 arginine mutation

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<220>
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<220>
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     corresponding to rat albumin promoter

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SUBSTITUTE SHEET (RULE 26)

-3-

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<210> 9
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<212> DNA
<213> Artificial Sequence

<220>
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<210> 10
<211> 91
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<213> Artificial Sequence

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cgaattccag ttctcacgtg gtggccacgt g                                91
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SUBSTITUTE SHEET (RULE 26)

-4-

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<220>
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      promoter and 5' untranslated region and human
      insulin

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attagagcga gtttctctgc acacagacca cctttcctgt caacccactg cctctggcac 240
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      promoter and 5' untranslated region and human
      insulin

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-5-

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<212> DNA
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<220>
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      insulin

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INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6   C12N15/63   C12N15/85   C12N15/17   C12N15/11   C07K14/62
         A61K48/00   A61K38/28   //A61P3/10,C12N15/861

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6   C12N   C07K   A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ALAM ET AL: "Glucose inducible expression of human insulin in hepatocytes" ACTA DIABETOLOGICA, vol. 34, no. 2, September 1997, page 115 XP002098527 see abstract	1-4,7-9
X	MUZZIN ET AL: "Hepatic Insulin Gene Expression as Treatment for Type 1 Diabetes Mellitus in Rats" MOLECULAR ENDOCRINOLOGY, vol. 11, no. 6, 1997, pages 833-837, XP002098528 see the whole document	1,3,4, 7-9

−/−−

| X | Further documents are listed in the continuation of box C. | | X | Patent family members are listed in annex. |

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
31 March 1999	13/04/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Jansen, K-S

Form PCT/ISA/210 (second sheet) (July 1992)

4

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate. of the relevant passages	Relevant to claim No.
X	SHIH ET AL: "Two CACGTG Motifs with Proper Spacing Dictate the Carbohydrate Regulation of Hepatic Gene Transcription" JOURNAL OF BIOLOGICAL CHEMSITRY, vol. 270, no. 37, 15 September 1995, pages 21991-21997, XP002098529 see abstract see discussion	5,6
A	VALERA ET AL: "Regulated expression of human insulin in the liver of transgenic mice corrects diabetic alterations" FASEB JOURNAL, vol. 8, 1994, pages 440-447, XP002098530 cited in the application see abstract	1-9
A	KOLODKA ET AL: "Gene therapy for diabetes mellitus in rats by hepatic expression of insulin" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES USA, vol. 92, April 1995, pages 3293-3297, XP002098531 see abstract	1-9
E	WO 98 31397 A (WISCONSIN ALUMNI RES FOUND) 23 July 1998 see the whole document	1-9

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INTERNATIONAL SEARCH REPORT

Box I   Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. **[X]** Claims Nos.:
   because they relate to subject matter not required to be searched by this Authority, namely:

   Remark: Although claims 8 and 9
            are directed to a method of treatment of the human/animal
            body, the search has been carried out and based on the alleged
            effects of the compound/composition.

2. ☐ Claims Nos.:
   because they relate to parts of the International Application that do not comply with the prescribed requirements to such
   an extent that no meaningful International Search can be carried out, specifically:

3. ☐ Claims Nos.:
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II   Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all
   searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment
   of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report
   covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is
   restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest   ☐ The additional search fees were accompanied by the applicant's protest.

                                ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Patent Document cited in search report		Publication date	Patent family member(s)	Publication date
WO 9831397	A	23-07-1998	AU 5926998 A	07-08-1998